ENVIRONMENT, WELL-BEING, AND BEHAVIOR

Reduction of *Salmonella enterica* Serovar Enteritidis Colonization and Invasion by an Alfalfa Diet During Molt in Leghorn Hens

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ABSTRACT The standard method for molting to stimulate multiple egg-laying cycles in laying hens is feed deprivation. However, the physiological changes within hens caused by feed deprivation increase susceptibility of the hens to *Salmonella enterica* serovar Enteritidis (SE) infection. In an effort to develop an alternative method to induce molting without increasing susceptibility to SE, an alfalfa diet was compared with the standard molting method for the level of ovary regression and SE colonization. Hens over 50 wk of age were divided into 3 treatment groups (12 hens/group): nonmolting by normal feeding (NM), molting by feed deprivation (M), and molting by alfalfa diet (A). Individual hens on all treatments were challenged orally with 10^5 cfu of SE on the fourth day after feed changes and were analyzed for ovary weight and SE colonization or invasion in crop contents, cecal contents, liver, spleen, and ovary on the ninth day. In 3 of the 4 trials, there was a significant decrease in SE colonization of the crop between the alfalfa diet (A) and the feed deprived molt (M). In most of the 4 trials, there was a significant reduction in SE infected organs in birds fed the alfalfa diet (A) compared with birds undergoing feed deprived molt (M). Most of the trials showed no significant difference in overall SE between A and NM. Therefore, the results of this study suggest that an alfalfa diet has the potential to be used as an alternative method for forced molting, without increasing the incidence of SE in eggs and internal organs.

(*Key words*: *Salmonella* enteritidis, molting, laying hen, alfalfa, feed withdrawal)

INTRODUCTION

Induced molting is used by the industry for optimal economical egg production (Bell, 2003). The most effective method of ovary regression and molt is feed withdrawal for 10 to 14 d (Bell, 2003). Molting by this method is very stressful and can increase bird mortality, although this mortality is most likely related to already weakened or diseased birds that will succumb to mortality shortly even without molting (Webster, 2003). This feed withdrawal behavior is actually normal in birds that are undergoing other activities that will take them away from food gathering and hunting such as nesting, mating, and molting. Wild jungle fowl hens will not eat for 20 d while nesting and penguins for up to 4 mo (Cherel et al., 1994; Webster, 2003). Some species even refuse feed until the last stages of molt (Mrosovsky and Sherry, 1980).

Some investigators have found that susceptibility to some diseases decrease during anorexia (Webster, 2003). Feed withdrawal molt, however, has been shown in several studies to increase bird's susceptibility to *Salmonella enterica* serovar Enterididitis (SE) infection (Holt, 1993; Corrier et al., 1997; Durant et al., 1999; Ricke, 2003). SE infection of a bird at this stage could lead to production of SE-positive eggs after molt (Humphrey et al., 1989). SE is a serious food safety concern worldwide. Although poultry is not the only source of this pathogen, it is the most common food source (St. Louis, 1988; Patrick et al., 2004). The most prevalent source of SE infection of humans when the origin can be identified is eggs (Patrick et al., 2004). The major concern with SE is that it has a transovarian route of infection, resulting in direct deposition into the yolk from an infected laying hen and thus can bypass egg washes and disinfectants of the intact egg (Humphrey et al., 1989; Gast and Beard, 1993; Thiagarajan et al., 1994).

Abbreviation Key: A = alfalfa treatment; BGA = brilliant green agar; M = feed withdrawal treatment; NM = nonmolted treatment; NO-NA = novobiocin and naladixic acid; SE = *Salmonella enterica* serovar Enteritidis; TT = tetrathionate broth; VFA = volatile fatty acid.

Alternative molting diets are becoming a popular alternative to feed withdrawal molt, but it still remains to be seen whether these alternative diets are truly more beneficial to the health and well-being of birds (Webster, 2003). Many alternative molting diets have been devised and work well for ovarian regression required for optimal postmolt egg production, although some diets work better than others. The diets are based on general nutrient limitation, mineral limitation/depletion, ingredients that decrease appetite, and addition of fillers or administration of hormones (Bell, 2003; Holt, 2003; Park et al., 2004). Less is known regarding the ability of these alternative molting diets to restrict SE colonization and infection. The majority of the work has been focused on wheat middlings and the zinc diets (Seo et al., 2001; Moore et al., 2004).

Alfalfa is the primary and first-used forage crop in the world; its use predates recorded history (Hanson, 1972). At the turn of the century and in some parts of the world today, laying hens are turned out in alfalfa crops as their only source of food, which of course would include insects and worms also found in alfalfa field (Wing, 1912; Hanson, 1972). Alfalfa is currently added to chicken feed for the xanthophylls that are used to color skin and egg yolk. It is high in protein, vitamins, and minerals. There are some inhibitory effects to growth of chicks at high concentrations (German and Couch, 1950), and thus alfalfa has not been used as a primary protein source. Alfalfa in high concentrations in the diet of layers decreases growth and egg production (Heywang, 1950). Landers et al. (2005b) have shown alfalfa as a single dietary source to be another alternative molting diet that effectively causes ovary regression and retains initial postmolt egg production responses comparable with that of feed withdrawal. Landers et al. (2005a) have shown that alfalfa as an alternative molt induction diet does not significantly change the color of the yolk or taste as rated by a sensory panel. They did, however, find a significant difference in yolk color of chickens fed alfalfa when examined using Minolta Colorimetry instrumentation. We examined alfalfa as an alternative diet to potentially reduce the incidence of SE infection in molting laying hens by examining SE-infected hens and fermentation responses.

MATERIALS AND METHODS

Bacteria

A primary poultry isolate of SE (phage type 13A) obtained from the National Veterinary Services Laboratory,[4] was selected for resistance to novobiocin (NO) and nalidixic acid (NA) in the Agricultural Research Service Food Animal Protection Research Laboratory and maintained on nutrient agar. The medium used to culture the resistant isolate in experimental studies contained 25 μg of NO and 20 μg of NA/mL. The challenge inocula were prepared from an overnight culture that had been previously transferred 3 times in trypticase soy broth. The culture was serially diluted in sterile phosphate-buffered saline to a concentration of approximately 10^5 cfu/mL. The colony-forming units of the challenge inoculum were confirmed by plating onto brilliant green agar (BGA).[5]

Molt Procedure

Hens were molted by a modification (Holt et al., 1995) of a previously described procedure (Brake et al., 1982). Seven days before feed removal, hens were exposed to a photoperiod of 8L:16D, which was continued throughout the experiment. Beginning on d 0, feed was withdrawn for 9 d for the (M) group, fed ad libitum feed to the nonmolted control (NM), and fed 100% alfalfa (A) ad libitum for 9 d, after which the study was ended.

Experimental Protocol

Single comb White Leghorn hens[6] over 50 wk of age were obtained from a commercial laying flock. Clocal swab samples were collected from each hen and examined for salmonellae by successive culturing in tetrathionate (TT)[5] broth and BGA as described by Andrews et al. (1992). All *Salmonella*-positive hens were removed before the study. The hens were placed in wire layer cages (2 hens per cage) and provided free access to water throughout the study and a balanced unmedicated corn-soyabean-meal based mash layer feed ration that met National Research Council requirements for nutrients (NRC, 1994). This diet provided 2,818 kcal of ME/kg, 16.5% CP, 3.5% calcium, and 0.48% available phosphorus. Before use, 3 randomly selected 25-g samples of the feed were cultured successively in buffered peptone water, TT broth, and BGA as described by Andrews et al. (1992) and were examined for salmonellae. *Salmonella* was not detected in the feed samples. The hens were allowed to acclimate for 1 wk and then were randomly assigned to 3 treatment groups of 12 hens each designated as either unmolted controls, molted by feed withdrawal, or molted by 100% alfalfa. The unmolted controls were given ad libitum access to the balance layer ration and the molted by alfalfa birds are given ad libitum access to 100% powdered alfalfa meal.[7] The hens were housed at USDA-ARS (College Station, TX) approved facilities under a protocol approved by the USDA-ARS Animal Use and Care Committee.

On d 4 of molt, all hens in each group were challenged by crop gavage with 1 mL of inoculum containing approximately 10^5 cfu of NA-NO-resistant SE. The challenge dosage was approximately equal to the 5.6×10^4 cfu dosage reported previously to be the mean infectious dosage (ID_{50}) for SE in unmolted hens (Holt, 1993).

On d 9 of molt, hens from each group were euthanatized, and crop pH was determined as reported by Durant et al. (1999). Each crop was excised and cut open asep-

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[4]Ames, IA.
[5]Difco Laboratories, Detroit, MI.
[6]Hyline International, Bryan, TX.
[7]Producers Cooperative, Bryan, TX.

tically, and the entire crop and contents together with 10 mL of sterile distilled water were blended for 1 min.[8] Samples of the blended crop were analyzed for concentrations of lactic acid. The crop, ceca, liver, spleen, and ovaries were excised aseptically and cultured separately for SE. Cecal samples were saved also for lactic acid and volatile fatty acid (VFA) analyses. The experimental protocol was repeated in 4 separate trials using hens obtained from the same commercial flock.

VFA and Lactic Acid Concentrations

The concentrations of VFA in the cecal contents were determined by gas-liquid chromatography as described by Corrier et al. (1990). Briefly, the analyses were conducted with a gas chromatograph equipped with a flame ionization detector and peak profiles integration-quantification integrator.[9] Each sample peak profile was integrated and quantified relative to an internal standard of methylbutyric acid placed in the same sample. Analyses were conducted at an oven temperature of 200°C and a flow rate of 85 mL/min. The concentration of each acid was expressed as micromoles per milliliter. Lactic acid concentrations were determined by an enzymatic method (Hohorst, 1965).

Crop Colonization by SE

One milliliter of the blended crop sample was transferred into 10 mL of TT broth and incubated for 24 h at 37°C. After incubation, the broth was streaked onto NO-NA-BGA plates, incubated for an additional 24 h at 37°C, and examined for the presence of suspect SE colonies. Suspect colonies were confirmed by biochemical tests on triple sugar-iron agar and lysine-iron agar[10] and further identified as SE serologically using *Salmonella* O antiserum group D, factors 1, 9, and 12.[5] Identification of the NO-NA-resistant-SE by the culture on NO-NA-BGA plates and by the biochemical and serological procedures described was considered confirmatory without further serotyping.

Cecal Colonization by SE

One cecum from each hen was cut into several pieces, placed in 30 mL of TT broth, shaken vigorously, and incubated for 24 h at 37°C. After incubation, the broth was streaked on NO-NA-BGA plates, incubated for an additional 24 h at 37°C, and examined for the presence of suspect SE colonies. Suspect colonies were confirmed biochemically and serologically as described in the previous section.

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[8]Stomacher 80 Lab Blender, Stewart Medical, London, England.
[9]Schimadzu Corp., Columbia, MD.
[10]Oxoid, Unipath Ltd., Hampshire, England.
[11]Biotran III, New Brunswick Scientific Co., Edison, NJ.
[12]SAS Institute, Cary, NC.

SE Per Gram of Cecal Content

The contents of one cecum from each hen were serially diluted (10^{-1} through 10^{-4}) and spread plated on NO-NA-BGA plates. The plates were incubated for 24 h at 37°C, and the number of colony-forming units of SE per gram of cecal contents were determined with an automatic colony counter.[11] SE colonies were confirmed biochemically and serologically as described above. Cecal contents in which SE were not detected at the 10^{-1} dilution on BGA plates or after TT broth enrichment and BGA plating were scored as 0 cfu. Cecal contents that were negative at 10^{-1} dilution on BGA plates but were positive after TT enrichment and BGA plating were arbitrarily assigned log 0.95 cfu of SE per gram of cecal contents.

Liver, Spleen, and Ovary Colonization by SE

Liver, spleen, and ovary specimens were minced with scissors, and samples were incubated for 24 h at 37°C in TT broth. After incubation, the broth was streaked onto NO-NA-BGA plates, incubated for an additional 24 h at 37°C, and examined for the presence of SE colonies. Suspect colonies were confirmed biochemically and serologically as described previously.

Statistical Analysis

Chi-squared analysis was used to determine significant differences among treatment groups for incidences of SE colonization of the crop, cecal, liver, spleen, and ovary (Luginbuke and Schlotzhauer, 1987). Differences in the cecal pH, VFA, lactic acid concentrations, and log colony-forming units of SE counts among treatment groups were determined by ANOVA using the GLM procedures. Significant differences were further separated using Duncan's multiple range test and commercial statistical analysis software.[12] All data were analyzed by individual trial. All statistical analyses were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Laying Hen Response to Treatments

The feed intake in trials 2, 3, and 4 (Table 1) exhibited significant differences between the full fed group and the alfalfa diet with as much as 5-fold less in 2 of the trials. Water intake was calculated per milliliter per hen daily, and the full fed group was significantly higher (at least 2-fold) for water intake that the other 2 treatments in 3 of the 4 trials. In 3 of the 4 trials (trials 1, 2, and 3), alfalfa diet and nonfed molt control did not exhibit significant differences in water intake.

The change in BW over the trial period decreased significantly in the molted and alfalfa molted diets compared with the nonmolted control as would be expected. There was a significant decrease in molted control than alfalfa

TABLE 1. Effects of nonmolting and molting with and without alfalfa on feed intake,
body weight loss or gain, and ovary weight of hens

Item		Treatment		
		NM[1]	M[2]	A[3]
Trial 1	Feed intake (g/hen daily)	Not done	Not done	Not done
	Water intake (mL/hen daily)	211.09 ± 10.40[a]	101.83 ± 7.58[b]	96.64 ± 13.56[b]
	Body weight loss or gain (g)	−49.0 ± 25.20[a]	−370.50 ± 53.00[b]	−357.08 ± 42.22[b]
	Ovarian weight[4]	2.26 ± 0.369[a]	0.58 ± 0.06[b]	0.38 ± 0.04[b]
Trial 2	Feed intake (g/hen daily)	86.23 ± 3.03[a]	NA	15.31 ± 2.89[b]
	Water intake (mL/hen daily)	196.26 ± 5.39[a]	109.22 ± 9.26[b]	90.71 ± 4.90[b]
	Body weight loss or gain (g)	−30.33 ± 22.57[a]	−456.00 ± 28.23[c]	−362.67 ± 34.36[b]
	Ovarian weight[4]	2.72 ± 0.32[a]	0.47 ± 0.06[b]	0.34 ± 0.03[b]
Trial 3	Feed intake (g/hen daily)	87.21 ± 4.17[a]	NA	17.06 ± 1.39[b]
	Water intake (mL/hen daily)	183.28 ± 6.80[a]	75.90 ± 6.28[b]	68.49 ± 7.58[b]
	Body weight loss or gain (g)	42.50 ± 34.63[a]	−358.58 ± 61.23[b]	−328.33 ± 25.93[b]
	Ovarian weight[4]	2.76 ± 0.38[a]	0.55 ± 0.06[b]	0.50 ± 0.03[b]
Trial 4	Feed intake (g/hen daily)	76.57 ± 3.17[a]	NA	20.21 ± 3.14[b]
	Water intake (mL/hen daily)	162.91 ± 8.23[a]	76.16 ± 30.19[b]	116.62 ± 14.70[ab]
	Body weight loss or gain (g)	−45.42 ± 37.17[a]	−513.33 ± 19.35[c]	−360.08 ± 40.76[b]
	Ovarian weight[4]	2.22 ± 0.33[a]	0.63 ± 0.13[b]	0.54 ± 0.06[b]

[a–c]Means within a row with no common superscripts differ significantly ($P < 0.05$).

[1]NM = nonmolted hens that received a Texas A&M University (TAMU) layer ration for 9 d.

[2]M = molted hens that were undergoing feed withdrawal for 9 d.

[3]A = hens that received 100% alfalfa for 9 d during molt.

[4]As a percentage of body weight: (ovary weight/body weight) × 100.

[5]NA = not applicable.

diet in 2 of the 4 trials (trial 2 and 4). This finding is similar to results from Landers et al. (2005b), who demonstrated a greater decrease in BW in the molted treatment and the A treatment. It has been shown that approximately a 30% reduction in BW is needed for a successful ovary regression (Baker et al., 1983).

The ovary weights of the birds are calculated as a percentage of their BW. In all 4 trials, there was a significant decrease in the 2 molted treatments compared with the nonmolted control. There was no significant difference in ovary weights between the 2 molted treatments. The results are similar to the ovary reduction responses observed by Landers et al. (2005b).

Crop pH and Lactic Acid

Crop pH and lactic acid concentrations were taken at the end of the trials (Table 2). In all trials, the nonmolted control was significantly lower in pH that the other 2 treatments. These results are similar to the pH of the NM and M treatments reported by Durant et al. (1999) and Moore et al. (2004). In 3 of the 4 trials (trials 1, 2, and 4), there was no difference between the pH of a feed withdrawal group and an alfalfa group. In 3 of the 4 trials, lactic acid concentrations were measured. Lactic acid was significantly higher in the crops of nonmolted birds than the 2 molted groups in only trial 3. The other 2 trials exhibited some variation. In general, the bird crops from the NM treatment yielded higher levels of lactic acid than crop levels from molted birds. Moore et al. (2004) also reported a significant difference between the NM and M treatments.

SE Colonization of the Crop

Enumeration of SE colonization of the crop is shown in Table 3. In all the trials, in the nonmolted group, SE were not detected in the crop. Based on chi-squared analysis there were no differences between colonization of the crop in the 2 molting treatments and the full fed control. With regard to colony-forming units per, there were no differences among treatments except in trial 4 feed withdrawal molt and the alfalfa treatment. It has been demonstrated that lactate inhibits *Salmonella* in vitro growth and is more effective in the pH ranges of the crop found in the nonmolted birds than in the pH range of the molted treatments (Hinton et al., 1993; McHan and Shotts, 1993).

SE Colonization of the Ceca

The results of SE colonization in the ceca are given in Table 4. Based on chi-squared analysis, there were no differences between the alfalfa diet and full fed control in cecal enrichment positives in 3 of the 4 trials. In 3 of the 4 trials, the birds in the feed withdrawal treatment yielded significantly higher levels of SE colony-forming units per gram than the other 2 treatments, which did not differ. Only in trial 3 was there no difference among all treatments with regard to SE colony-forming units per gram. In general there was a significant increase in the proportion of SE-positive ceca in birds receiving the M treatments when compared with the NM birds, which was also similar to the response observed by Durant et al. (1999), Moore et al. (2004) and Seo et al. (2001).

TABLE 2. Effects of nonmolting and molting with or without alfalfa on crop pH and lactic acid concentrations

Item	Treatment		
	NM[1]	M[2]	A[3]
Trial 1			
Crop pH	4.64 ± 0.16^b	5.86 ± 0.08^a	5.73 ± 0.14^a
Lactic acid (mmol/mL)	82.67 ± 5.98^a	82.24 ± 18.27^a	24.82 ± 2.96^b
Trial 2			
Crop pH	4.50 ± 0.08^b	5.34 ± 0.19^a	5.58 ± 0.17^a
Lactic acid (mmol/mL)	57.04 ± 3.29^a	19.55 ± 1.15^b	42.63 ± 10.86^a
Trial 3			
Crop pH	4.65 ± 0.12^c	5.80 ± 0.14^a	5.34 ± 0.17^b
Lactic acid (mmol/mL)	41.86 ± 4.60^a	11.62 ± 2.36^b	9.23 ± 1.23^b
Trial 4			
Crop pH	4.07 ± 0.12^b	5.03 ± 0.07^a	4.69 ± 0.17^a
Lactic acid (μmol/mL)	Not done	Not done	Not done

[a–c]Means within a row with no common superscripts differ significantly ($P < 0.05$).

[1]NM = nonmolted hens that received a Texas A&M University (TAMU) layer ration for 9 d.

[2]M = molted hens that were undergoing feed withdrawal for 9 d.

[3]A = hens that received 100% alfalfa for 9 d during molt.

SE in the Organs

The results of SE enrichments on the liver, spleen, and ovaries are shown in Table 5. The livers from birds in trials 2 and 4 exhibited significant differences between enrichment SE-positive birds undergoing feed withdrawal compared with the other 2 treatments. In trial 1, there was a significant difference between feed withdrawal and the nonmolted control but not with birds fed alfalfa. In trial 3, a significant decrease in SE positive birds was observed between the nonmolted group and the other 2 treatments, but there was no difference between the molt treatments.

The enriched spleen samples that were SE positive (Table 5) showed a significant increase in the feed withdrawal molt over the nonmolted control and the alfalfa group, which showed no significant difference between each other in trials 1, 2, and 4. In trial 3, there was no significant difference between birds fed alfalfa and the other 2 groups, but there was still a significant difference between birds undergoing feed withdrawal molt and the nonmolted control birds.

In trial 2, there was no difference in ovary SE-positive enrichments (Table 5) between the 3 treatments. A significant increase in SE-positive ovaries from birds undergoing molted treatments compared with the nonmolted control birds was observed for trial 1, whereas a significant increase in SE-positive ovaries in the feed withdrawal group from the nonmolted control but not from alfalfa was observed for trial 3. There was a significant increase in SE-positive ovaries in the feed withdrawal molt over the other 2 treatments in trial 4.

TABLE 3. Effects of nonmolting and molting with and without alfalfa on *Salmonella entericia* serovar Entertidis (SE) crop colonization of hens

Item	Treatment[1]		
	NM[2]	M[3]	A[4]
Trial 1			
Positive hens per total (%)	$0/12\ (0\%)^a$	$1/12\ (8.33\%)^a$	$2/12\ (16.67\%)^a$
Log_{10} cfu/g	0^a	0.17 ± 0.17^a	0^a
Trial 2			
Positive hens per total (%)	$0/12\ (0\%)^a$	$3/12\ (25\%)^a$	$2/12\ (16.67\%)^a$
Log_{10} cfu/g	0^a	0.67 ± 0.36^a	0.51 ± 0.35^a
Trial 3			
Positive hens per total (%)	$0/12\ (0\%)^a$	$2/12\ (16.67\%)^a$	$3/12\ (25\%)^a$
Log_{10} cfu/g	0^a	0.51 ± 0.37^a	0.64 ± 0.37^a
Trial 4			
Positive hens per total (%)	$0/12\ (0\%)^a$	$4/12\ (33.33\%)^a$	$1/12\ (8.33\%)^a$
Log_{10} cfu/g	0^b	1.10 ± 0.52^a	0.23 ± 0.23^{ab}

[a–c]Means within a row with no common superscripts differ significantly ($P < 0.05$).

[1]Hens were challenged by crop gavage with 10^5 cfu of SE on d 4 of molt and cultured for *Salmonella* on d 9 of molt.

[2]NM = nonmolted hens that received a Texas A&M University (TAMU) layer ration for 9 d.

[3]M = molted hens that were undergoing feed withdrawal for 9 d.

[4]A = hens that received 100% alfalfa for 9 d during molt.

**TABLE 4. Effects of nonmolting and molting with and without alfalfa on *Salmonella entericia*
serovar Entertidis (SE) cecal colonization of hens**

	Treatment[1]		
Item	NM[2]	M[3]	A[4]
Trial 1			
Positive hens per total (%)	2/12 (16.67%)[b]	7/12 (58.33%)[a]	0/12 (0%)[b]
Log_{10} cfu/g	0.52 ± 0.35[b]	3.39 ± 0.90[a]	1.05 ± 0.7[b]
Trial 2			
Positive hens per total (%)	1/12 (8.33%)[b]	12/12 (100)[a]	4/12 (33.33%)[b]
Log_{10} cfu/g	0.19 ± 0.19[b]	4.81 ± 0.55[a]	1.37 ± 0.60[b]
Trial 3			
Positive hens per total (%)	2/12 (16.67%)[b]	7/12 (58.33%)[a]	7/12 (58.33%)[a]
Log_{10} cfu/g	0.79 ± 0.6[a]	2.96 ± 0.82[a]	2.65 ± 0.74[ab]
Trial 4			
Positive hens per total (%)	1/12 (8.33%)[b]	9/12 (75%)[a]	3/12 (25%)[b]
Log_{10} cfu/g	0.23 ± 0.23[b]	4.50 ± 0.85[a]	1.03 ± 0.56[b]

[a,b]Means within a row with no common superscripts differ significantly ($P < 0.05$).

[1]Hens were challenged by crop gavage with 10^5 cfu of SE on d 4 of molt and cultured for *Salmonella* on d 9 of molt.

[2]NM = nonmolted hens that received a Texas A&M University (TAMU) layer ration for 9 d.

[3]M, Molted = hens that were undergoing feed withdrawal for 9 d.

[4]A = hens that received 100% alfalfa for 9 d during molt.

Durant et al. (1999), Moore et al. (2004), and Seo et al. (2001) have also reported a significant increase in organ invasion in molted birds when compared with non-molted controls.

Cecal VFA Profile of Controls and Alfalfa Diet

There appears to be a connection, at least indirectly, between *Salmonella* establishment and concentrations of various cecal VFA (Corrier et al., 1990; Nisbet et al.,

1996; van der Wielen et al., 2001). VFA have been shown to change the invasiveness of *Salmonella* to cecal epithelial cells in vitro (van Immerseel et al., 2004). Concentrations of VFA similar to those found in the ceca have been shown to inhibit the growth of *Salmonella*; this inhibition is increased with the reduction in redox potential of the ceca accompanied by a lower pH of the ceca (Meynell, 1963; Royal and Mutimer, 1972; Barnes et al., 1979; Hinton et al. 1993; McHan and Shotts, 1993). Donalson et al. (2004) demonstrated the reduction in *Salmonella* was equivalent in vitro when incubations of

**TABLE 5. Effects of nonmolting and molting with and without alfalfa on *Salmonella entericia* serovar
Entertidis (SE) colonization of the liver, spleen, and ovary of hens**

|	Treatment[1]		
Item	NM[2]	M[3]	A[4]
Trial 1			
Liver	0/12 (0%)[b]	5/12 (41.67%)[a]	2/12 (16.67%)[ab]
Spleen	0/12 (0%)[b]	7/12 (58.33%)[a]	2/12 (16.67%)[b]
Ovary	0/12 (0%)[b]	8/12 (66.67%)[a]	4/12 (33.33%)[a]
Trial 2			
Liver	4/12 (33.33%)[b]	10/12 (83.33%)[a]	4/12 (33.33%)[b]
Spleen	1/12 (8.33%)[b]	7/12 (58.33%)[a]	2/12 (16.67%)[b]
Ovary	1/12 (8.33%)[a]	4/12 (33.33%)[a]	1/12 (8.33%)[a]
Trial 3			
Liver	0/12 (0%)[b]	8/12 (66.67%)[a]	6/12 (50%)[a]
Spleen	0/12 (0%)[b]	4/12 (33.33%)[a]	6/12 (50%)[ab]
Ovary	1/12 (8.33%)[b]	6/12 (50%)[a]	3/12 (25%)[ab]
Trial 4			
Liver	0/12 (0%)[b]	8/12 (66.67%)[a]	1/12 (8.33%)[b]
Spleen	0/12 (0%)[b]	8/12 (66.67%)[a]	2/12 (16.67%)[b]
Ovary	0/12 (0%)[b]	10/12 (83.33%)[a]	0/12 (0%)[b]

[a–c]Means within a row with no common superscripts differ significantly ($P < 0.05$).

[1]Hens were challenged by crop gavage with 10^5 cfu of SE on d 4 of molt and cultured for *Salmonella* on d 9 of molt.

[2]NM = nonmolted hens that received a Texas A&M University (TAMU) layer ration for 9 d.

[3]M = molted hens that were undergoing feed withdrawal for 9 d.

[4]A = hens that received 100% alfalfa for 9 d during molt.
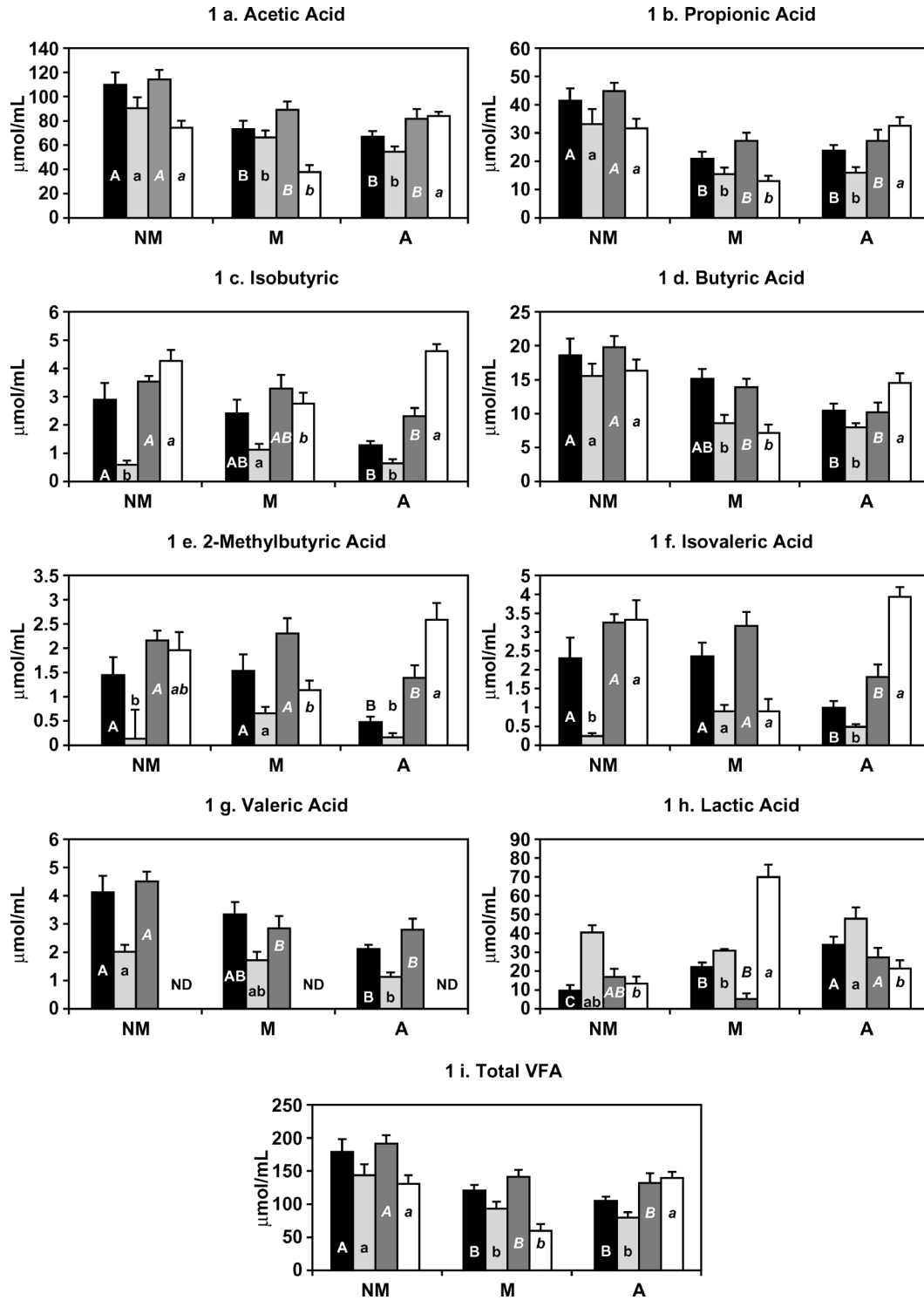
FIGURE 1. Effects of nonmolting and molting with and without alfalfa on cecal volatile fatty acids (VFA) and lactic acid concentrations (μmol/mL). [A–C]Means within trial 1 without a common letter differ significantly ($P < 0.05$); [a–c]Means within trial 2 without a common letter differ significantly ($P < 0.05$); [A–C]Means within trial 3 without a common letter differ significantly ($P < 0.05$); [a–c]Means within trial 4 without a common letter differ significantly ($P < 0.05$). NM = nonmolted hens that received a Texas A&M University (TAMU) layer ration for 9 d; M = molted hens that were undergoing feed withdrawal for 9 d; A = hens that received 100% alfalfa for 9 d during molt.

layer ration and alfalfa as substrates were mixed with cecal contents.

The VFA concentrations were compared from cecal contents in the 4 trials (Figure 1). The acetic acid concentrations are given in Figure 1a. In trials 1, 2, and 3, acetic

acid was significantly higher in the nonmolted control than the 2 molted treatments, which were not significantly different from each other. Propionic acid (Figure 1b) exhibited a pattern similar to that of acetic acid with respect to the individual trials. In general, ceca from

birds on the 2 molted treatments exhibited greater declines in acetic and propionic acid than ceca from birds on the nonmolted treatment. These trends in the comparison between nonmolted and feed withdrawal molt birds were similar to those reported by Corrier et al. (1997).

Isobutyric acid concentrations (Figure 1c) were variable among trials. In 2 of the 4 trials (trials 2 and 3), butyric acid (Figure 1d.) was significantly higher in concentration in the nonmolted treatment than the 2 molting treatments, which were not significantly different from each other.

The 2-methylbutyric acid concentrations are presented in Figure 1e. Significantly higher concentrations were observed in the nonmolted and feed withdrawal molt birds than in the alfalfa molt birds in 2 of the 4 trials (1 and 3). In trials 1 and 3, isovaleric acid concentration (Figure 1f.) was significantly higher in the nonmolted control and the feed withdrawal molt treatment than the alfalfa group. The concentrations of valeric acid are shown in Figure 1g. The nonmolted control has significantly higher concentrations of valeric acid than the alfalfa group but not the feed withdrawal group in trials 1 and 2. In trial 3, the nonmolted group was significantly higher than the 2 molted treatments, which were not different from each other. The concentrations of lactic acid are shown in Figure 1h. Birds undergoing alfalfa molt yielded significantly higher levels of cecal lactic acid than the feed withdrawal molt group but not the nonmolted control treatment in trials 2 and 3.

The total VFA concentrations are shown in Figure 1i. In 3 of the 4 trials (trials 1, 2, and 3), birds from the nonmolted control group yielded significantly higher concentrations of cecal VFA than the feed withdrawal molt birds (similar to Corrier et al., 1997) and the alfalfa molt birds, which were not different from each other. If concentrations of VFA in the ceca reduce SE colonization of the ceca, then it would appear that, in general, alfalfa would be more similar to the feed withdrawal molt than nonmolting birds when limiting invasion. The general increase in lactic acid in the ceca of birds on alfalfa treatment vs. those on feed withdrawal might be an indicator of why the alfalfa treatment was more inhibitory than the feed withdrawal molt. This finding was, however, not consistent among all of the 4 trials when comparing lactic acid concentration ranges with the high and low ranges of SE colonization. Therefore, VFA concentrations are probably not the only factor involved in the resistance to SE infection during alfalfa molt induction.

In conclusion, several molting diets have been developed that work well to achieve ovarian reduction (Bell, 2003; Holt, 2003; Park et al., 2004), and some also have been tested in their response to a *Salmonella* challenge (Seo et al., 2001; Moore et al., 2004). The alfalfa molting diet is an additional alternative diet that can be used by the poultry industry for effective ovary regression and provides some limitation to SE colonization of the hen ceca during stress associated with molt induction.

The goal of this diet was to achieve comparable ovarian reduction to the bird as compared with the conventional feed-withdrawal molt and examine whether dietary intake of alfalfa would increase the resistance to SE during molt by achieving gut fill that has been suggested to aid in the reduction of SE (Seo et al., 2001). For most of the trials SE reduction was accomplished. The lack of consistency for some of the parameter measured among the trials and incomplete elimination of SE in all trials may be due to the decrease in the amount of feed intake with alfalfa as well as the general decrease in VFA production compared with the full fed treatment. Alfalfa contains ingredients such as saponins that if present in high concentrations have been shown to cause a decrease in chick growth and reduce egg production in hens (Hanson, 1972). Corrier et al. (1990) and (1997) showed an increase in VFA and protection of chicks from *Salmonella* with the addition of lactose but a decrease in VFA when molting hens were given lactose in the drinking water. This did not however reduce the effectiveness against *Salmonella* of lactose in the drinking water. Seo et al. (2001), however, found no additional benefit to lactose addition to the wheat middling molting diet, but Donalson et al. (2004) showed that addition of fructooligosaccharide in vitro yielded similar reductions in *Salmonella* levels in the presence of layer ration or alfalfa incubated with cecal microbial inoculum. This finding suggests that addition of highly fermentable compounds such as lactose or fructooligaosaccharide to the alfalfa diet may increase its in vivo effectiveness against *Salmonella*. Likewise, mixing layer feed with alfalfa may also contribute to fermentation and help to minimize the problem of lowered feed intake (Moore et al., 2003).

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